Paternal-specific S-allele transmission in sweet cherry (Prunus avium L.): the potential for sexual selection

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Paternal-specific *S*-allele transmission in sweet cherry (*Prunus avium* L.): the potential for sexual selection.

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Abstract

Homomorphic self-incompatibility is a well-studied example of a physiological process that is thought to increase population diversity and reduce the expression of inbreeding depression. Whereas theoretical models predict the presence of a large number of *S*-haplotypes with equal frequencies at equilibrium, unequal allele frequencies have been repeatedly reported and attributed to sampling effects, population structure, demographic perturbation, sheltered deleterious mutations or selection pressure on linked genes. However, it is unclear to what extent unequal segregations are the results of gametophytic or sexual selection. While these two forces are difficult to disentangle, testing *S*-alleles in the offspring of controlled crosses provides an opportunity to separate these two phenomena. In this work, segregation and transmission of *S*-alleles have been characterised in progenies of mixed-donors and fully compatible pollinations under field conditions in *Prunus avium*. Seed set patterns and pollen performance have also been characterised. The results reveal paternal-specific distorted transmission of *S*-alleles in most of the crosses. Interestingly, *S*-allele segregation within any given paternal or maternal *S*-locus was random. Observations on pollen germination, pollen tube growth rate, pollen tube cohort size, seed set dynamics, and transmission patterns strongly suggest post-pollination, pre-zygotic sexual selection, with male-male competition as the most likely mechanism. According to these results, post-pollination sexual selection takes precedence over frequency dependent selection in explaining unequal S-haplotype frequencies.

Keywords:

Compatible mixed-donor pollination, transmission ratio distortion, *S*-allele transmission, pollen performance, post-pollination sexual selection.

INTRODUCTION

Homomorphic self-incompatibility (SI) is a well studied example of a plant physiological process governed by strong negative frequency dependent selection, in which rare alleles have a mating advantage compared to common ones (Uyenoyama, 2000). Negative frequency dependent selection is hypothesized to increase the number of alleles in the *S*-locus, and, in gametophytic SI systems, these alleles tend to have equal frequencies at equilibrium (Wright, 1939). Frequency expectation is more complex in sporophytic SI systems because of the added dominance relations between alleles; however, higher frequencies of recessive alleles are expected (Uyenoyama, 2000). Empirical evidence in support of equal frequencies have been found in natural populations of species with a gametophytic SI system (reviewed in Lawrence, 2000). However, deviations from equal frequencies have also been reported [e.g. *Papaver rhoeas*: (Campbell & Lawrence, 1981), *Prunus lannesiana* (Kato & Mukai, 2004), and *Prunus avium* (De Cuyper *et al.*, 2005; Stoeckel *et al.*, 2008)].

Mechanisms explaining these deviations range from characteristics at the organismal and population level, such as sampling effects, random genetic drift, population structure or demographic perturbations (Schierup, 1998), to selective processes acting at the genetic level and superimposed on negative frequency dependent selection at the *S*-locus (Bechsgaard *et al.*, 2004). These selective pressures might act on linked genes and can lead to transmission ratio distortion in single pollen donor pollinations. There is indeed evidence for transmission ratio distortion in plant SI alleles. Examples include species possessing sporophytic SI systems, such as *Arabidopsis lyrata* (Bechsgaard *et al.*, 2004). and species possessing gametophytic SI systems, such as *Papaver rhoeas* (Lawrence & Frankin-Tong, 1994) and *Prunus avium* (Ikeda *et al.*, 2005). Several mechanisms to explain these deviations, such as prezygotic meiotic drive, gametic or gametophytic selection, and postzygotic self-sterility and/or inbreeding depression, have been advocated (Bechsgaard *et al.*, 2004; Ikeda *et al.*,

2004; Wünsch & Hormaza, 2004a; Vilanova *et al.*, 2006; Leppälä *et al.*, 2008). Disentangling these alternatives is experimentally very demanding and few studies have addressed this question.

However, due to the alternation of generations, male gametophytic selection in plants can occur among haploid gametophytes either within a single pollen donor or among different pollen donors. The latter case occurs after mixed donors pollinations, which is likely to take place under natural conditions (e.g. Windsor *et al.*, 1998; Jolivet *et al.*, 2011), and is equivalent to sperm competition in animals in which gametes are produced directly after meiosis. Plant mating systems have been reported to depend on the quantity and identity of pollen grains reaching the stigma and on the ability of the female recipient to discriminate among them (Cruzan & Barret, 1996). Non-random mating after mixed donor pollinations has been extensively characterised in the literature (e.g. Rigney et al. 1993, Lankinen & Skogsmyr, 2002), and has been shown to lead to sexual selection whenever other confounding mechanisms of sorting among mates -such as self-incompatibility- could be excluded (e.g. Marshall & Ellstrand, 1986, Marshall 1998, Mitchel & Marshall, 1998, Marshall & Diggle, 2001; also see Skogsmyr & Lankinen, 2002 for a review on this controversial subject). It is unknown whether non-random mating or sexual selection occur in sweet cherry (*Prunus avium*) or in other species with a gametophytic self-incompatibility system, and whether they have any effect on *S*-allele segregation or transmission distortion.

While deviation from Mendelian segregation is usually assumed to be an inherent characteristic of the individual or interacting individuals (Ellstrand & Delvin, 1989), some works have unveiled the influence on gene frequencies across generations of environmental heterogeneity during gamete development and function as well as during early embryo development (reviewed in Hedhly *et al.*, 2009). Examples come from skewed paternity after mixed pollinations and under stressful environmental conditions [e.g. high temperature either

during the whole developmental cycle (Pasonen *et al.*, 2002) or restricted to the pollen developmental stage (Jóhannsson & Stephenson, 1998) and low temperature during the progamic phase [i.e. the period from pollination to fertilization (Linskens, 1986; Zamir & Tanksley, 1981)]. In *Raphanus sativus,* Ellstrand & Delvin (1989) found that heat stress bolstered deviant segregations by more than three times. However, whether environmental conditions might affect the transmission distortion of self-incompatibility alleles has not been addressed.

It is difficult to disentangle gametophytic from sexual selection. However, testing *S*-alleles in the offspring provides an opportunity to separate these two phenomena. Moreover, the use of clonal material, as is the case for sweet cherry cultivars, allows testing sexual selection on the same genotypes under different environments. In this work, we conducted a series of controlled single and mixed donor pollinations in sweet cherry (*Prunus avium* L.), during three years in the field. These crosses were fully cross-compatible and involved genotypes with contrasting flowering regimes and geographical origins. For the purpose of clarity, we considered random/distorted segregation as referring only to the sorting among gametophytes or gametes within a single pollen donor or of the female recipient (i.e. fair/unfair meiosis and random/distorted post-pollination transmission of the two alleles within a single parent irrespective of the pollination regime), whereas random/distorted transmission referring more generally to the post-pollination sorting among gametophyte or gametes of one or multiple pollen donors (transmission of any allele irrespective of the pollination regime). Mixed-donor pollination experiments were carried out to test (i) whether *S*-allele segregations of maternal and paternal donors were random/distorted under these competitive pollination regimes, (ii) whether they affect the post-pollination *S*-allele transmission to the seed, (iii) whether they might lead to sexual selection, and if so (iv) whether the genotype of the mother plant and the varying environmental conditions in different years might alter these transmission patterns.

MATERIALS AND METHODS

Plant material and mixed donor pollination experiments

Experiments were carried out in sweet cherry cultivar collections located at the Campus of Aula Dei in Zaragoza, Spain, with three clonally propagated individuals per cultivar. Eight sweet cherry cultivars (two female recipients and six pollen donors) were used for the pollination experiments (Table 1). These were chosen to design fully compatible crossings using *S*-haplotype information, and to maximize pollen donor differences (within the same mixed pollination) based on geographical origin and phenology.

Five crosses were performed along three consecutive years using two female recipients and four different pollen mixtures consisting of two pollen donors each (Table 1). The first year, we performed the cross 'Blanche de Provence' X (‘Cristobalina’ : ‘Vic’, 1:1 w/w pollen). The second year, and using the same female recipient trees, the cross ‘Blanche de Provence’ X (‘Burlat’ : ‘Royalton’, 1:1 w/w pollen) was carried out. The female recipient used in these two crosses was 'Blanche de Provence' (‘BPv’), a cultivar producing fruits with yellow-white coloured skin and flesh. As pollen donors we used two European traditional cultivars ('Cristobalina' and 'Burlat') and two cultivars resulting from breeding programs in North America ('Vic' and ‘Royalton’). The third year we repeated the cross of the first year together with two additional crosses: 'Blanche de Provence' X (‘Lambert’ : ‘Arcina’, 1:1 w/w pollen) and 'Talaguera Brillante' X (‘Cristobalina’ : ‘Vic’, 1:1 w/w pollen). The last two crosses include a new pollen mixture ('Lambert' and 'Arcina') that have the same paternal *S*-haplotypes found in ‘Vic’, ‘Cristobalina’ and ‘Royalton’ (S_2, S_3, S_4, S_6) but in a different combination, and an additional traditional European cultivar, ‘Talaguera Brillante’ (‘TBr’), used as a female recipient.

Pollen from each donor was obtained from flowers of three genetically identical clonal trees per genotype. For pollen collection, flowers were picked at balloon stage one day before anthesis (Baggiolini, 1952), and anthers were removed and let to dehisce at ambient temperature during 24 to 48 hours. Then, pollen was sieved through a 0.26 mm mesh to separate the pollen grains from the anther and filament debris and frozen at -20ºC until required. Like in many other species (Barnabás & Kovács, 1997), freezing sweet cherry pollen grain does not affect its function. The day before anthesis, flowers on the female recipient were synchronized by leaving only flower buds at balloon stage, on branches chosen randomly all over the trees, removing both open flowers or younger flower buds. The following day, thoroughly mixed equal quantities of pollen grains from a given pollen donor pair were prepared, and using brushes mildly loaded with pollen grains, pollination was carried out early in the morning just at flower opening and before anther dehiscence (Hedhly *et al.*, 2009). Daily maximum, minimum and average temperatures and relative humidity (Table 1), registered during the pollination-fertilisation phase (first week after pollination, Hedhly et al. 2007) were gathered from the nearby meteorological station of Montañana (Zaragoza, Spain).

Characterization of pollen performance and seed set dynamics

To study pollen performance during the progamic phase, flowers treated in the same way as above were pollinated in single pollination regimes during the three years of study. To check for possible differences in pollen performance between single and mixed donor pollinations regimes, an additional mixed donor pollination regime was also carried out the third year. A batch of 10 flowers per treatment was fixed daily in FAA [formalin: acetic acid: 70% ethanol, 1:1:18 v/v; (Johansen, 1940)] during the first 5 days following anthesis. Observations of

pollen germination and pollen tube growth were made on squashed stigma-styles washed three times in water, one hour each, autoclaved for 10 min at 1 kg/cm2 in 5% sodium sulphite (Jefferies & Belcher, 1974), and stained with 0.1% aniline blue in 0.1 N K3PO4 (Linskens & Esser, 1957; Hedhly *et al.*, 2005). Preparations were examined under a Leica DM2500 fluorescence microscope equipped with UV epifluorescence with a band pass BP 340-390 exciter filter and an LP 425 barrier filter (Leica Microsystems, Switzerland).

The numbers of adhered and germinated pollen grains were counted and the percentage of pollen germination was calculated. Depending on the cross, we recorded up to three parameters: the number of pollen tubes reaching different sections of style and ovary entrance, the length of the longest pollen tube (as a percentage of style length), and the percentage of flowers with pollen tubes at the base of their style. Besides reporting final seed set in all the crosses performed, flower and fruit drop were also monitored during the first two years (two crosses) by weekly counts on all tagged branches from anthesis to maturity. Relative drop, the percentage of flowers/fruitlets dropped each week, and initial and final seed set were then calculated.

_S_-locus analyses

At maturity, fruits from the different crosses were harvested. Sweet cherries produce fruits with a single seed, and embryos were isolated by removing tissues of maternal origin (pulp, endocarp and seed testa) and stored at -80ºC. DNA was extracted from the embryos according to a protocol described by Hormaza (2002) performing one to three additional phenol extractions. *S*-genotyping of the offspring was carried out by *S-RNase* typing by PCR following the procedure described by Sonneveld *et al.* (2006). The forward primer PaConsI-F (Sonneveld *et al.*, 2003) was labelled with WellRED fluorescent dyes D2, D3 and D4

(Proligo, Paris, France) and was used in combination with the reverse primer PaConsI-R2 (Sonneveld *et al.*, 2006). PCR amplification was carried out with 20 ng of genomic DNA in 15 µl reactions, which contained 16 mM (NH4)2SO4, 67 mM Tris-HCl pH 8.8, 0.01% Tween-20, 2.0 mM MgCl2, 0.1 mM dNTPs, 0.2 µM of each primer and 0.6 U BioTaq™ DNA polymerase (Bioline, London, UK). Amplification was carried out in a PTC-200 thermal cycler (MJ Research, Watertown, MA, USA) running the following cycling conditions: 2 min at 94 ºC, followed by 35 cycles of 1 min at 94 ºC, 1 min at 54ºC and 1 min at 72ºC, and a final extension step of 5 min at 72ºC. PCR amplification products were diluted as appropriate (D2 1.2 µl, D3 0.9 µl and D4 0.6 µl in 35 µl of formamide, and 0.45 µl 600 bp standard labeled with WellRed fluorescent dye D1), making single runs of 388 samples on a CEQ™ 8000 capillary DNA analysis system (Beckman Coulter, Fullerton, CA, USA). Results obtained from capillary electrophoresis were further checked by 1.5 % agarose gel electrophoresis as described in Wünsch & Hormaza (2004b) using consensus PruC2-PruC4 primers (Tao *et al.*, 1999), and a combination of the forward consensus primer PruT2 (Tao *et al.*, 1999) and reverse consensus primer SI32 (Wiersma *et al.*, 2001) and the specific primers, S_1, S_2, S_3, S_4, S_5, and S_6 (Sonneveld *et al.*, 2001; 2003). *S*-locus analysis on year 3 was performed by PCR using the primer pair PaConsI-F/PaConsIR2 for *S-RNase* genotyping (Sonneveld *et al.*, 2003; 2006). The forward primer was fluorescently labeled with 6-FAM, and PCR reactions were carried out according to Cachi & Wünsch (2014). In this case, PCR fragments were detected using ABI PRISM 3130xl genetic analyser (Applied Biosystems) and analysed with the Geneious software (Biomatters Ltd.). The Standard 500-GeneScan LIZ (Applied Biosystems) was used for fragment sizing.

Statistical and mathematical analyses

Two-way analyses of variance were carried out for testing differences in pollen germination percentages at the stigma level, for pollen tube growth along the style, and for the number of pollen tubes at the base of the style. All percentage data were subjected to arcsine root square transformation before the ANOVA analysis. The percentages of flowers with pollen tubes at the base of the style and final seed set were analysed using maximum likelihood G-test corrected for continuity in the case of 2x2 contingency tables (comparison with Pearson's Chi-squared test, with Pearson's Chi-squared Test with simulated p-value, and with Fisher's Exact Test for count data was carried out and no discrepancy was found). For *S*-allele segregation and transmission analyses, either G-test or replicated G-tests of goodness-of-fit (Sokal & Rohlf, 1995) were used for each year to examine deviation from the extrinsic hypothesis of random segregation (0.5 for each of the two *S*-alleles in a given locus, be it maternal or paternal), and random post-pollination transmission after mixed donor pollinations [expected transmission corrected for observed segregations: (observed paternal allele)*0.5 for each of the four paternal *S*-alleles, and ((observed maternal)*(observed paternal))*0.5 for each of the 8 offspring *S*-allele combinations)]. Degrees of Freedom *d.f* and number of observation *N* (can be a range *N-N* for pooled tests results) are given as subscripts: $G_{d.f,\ N}$.

Monitoring seed set rates and establishing seed paternity ratios prompted us to evaluate whether the knowledge of these two parameters allows to deduce the range in which zygotic paternity should lie. Using an algebraic analysis (see full reckoning within Supporting information Material and Methods) we established upper and lower boundaries in which zygotic paternity z should lie as a function of the seed set rate $\gamma \in [0,1]$ and the seed paternity ratio f (>0):

$$z_{min}(f, \gamma) \leq z(f, \gamma) \leq z_{max}(f, \gamma)$$

where z_{min} and z_{max} are given by

$$z_{max}(f,\gamma) = (f-\gamma+1)/\gamma$$

$$z_{min}(f,\gamma) = f\gamma/(1 + f - f\gamma)$$

For the purpose of testing zygotic bias, three major cases can be distinguished. The first is the case of a full seed set where we would theoretically expect that any bias in seed paternity should be a consequence of a bias in zygotic paternity. Indeed, for $\gamma = 1$, $z_{max} = z_{min} = z = f$. The second is the case of a non-biased seed paternity ($f = 1$). In this case the maximum and minimum values of zygotic paternity are

$z_{max} = (2-\gamma)/\gamma \geq 1$, (minimal value of 1 is reached when $\gamma = 1$)

$z_{min} = \gamma/(2-\gamma) \leq 1$, (maximal value of 1 is reached when $\gamma = 1$)

meaning that we cannot exclude an unbiased zygotic ratio ($z = 1$) for any value of seed set. The third is the case of a biased seed paternity ($f \neq 1$). A simple test for zygotic bias ($z \neq 1$) is to check if $z_{max} < 1$ or $z_{min} > 1$ holds true. In this case the test of zygotic bias depends on the values of f.

If $f > 1$: test if $z_{min} > 1$, replace within Eq. 15, test if $f > 1/(2\gamma-1)$

If $f < 1$: test if $z_{max} < 1$, replace within Eq. 14, test if $f < (2\gamma-1)$

z_{min} and z_{max} are plotted together as a function of γ for some given values of f.

R 3.2.2 (The R Foundation for Statistical Computing, 2004-2013; http://www.R-project.org) was used in statistical analyses and the R-package ggplot2 1.0.1 (Wickham, 2009) was used for graphical outputs. Part of the algebraic analysis was done with SymPy

0.7.6 (SymPy Development Team, 2014, SymPy: Python library for symbolic mathematics, http://www.sympy.org/) in Python 2.7.10.

RESULTS

S-allele transmissions after different mixed pollinations

In all crosses, we were unable to discriminate maternal alleles in 23 out of the 813 embryos analysed. Since these embryos were probably contaminated with maternal tissues, likely with testa remnants, we removed them from the final analysis.

S-allele typing in the offspring of the mixed-pollen donor crosses carried out the first two years (Year 1 and Year 2) revealed a random segregation of S-alleles within each genotype be it maternal or paternal, and a distorted paternal-specific post-pollination transmission of the four paternal alleles (Fig. 1, upper two rows). G-tests indicate that segregations of maternal alleles (S_1 vs. S_5 for the two crosses), and of paternal alleles within each pollen donor (S_2 vs. S_4, S_3 vs. S_6, and S_3 vs. S_9) were random and unaffected by pollen mixture or environmental conditions (maternal alleles: $G_{1,\ 204\text{-}238}$ range = 0.07-0.18, P range = 0.67-0.80; paternal alleles: $G_{1,\ 20\text{-}209}$ range = 0.00-2.60, P range = 0.11-1). However, the post-pollination transmission of the four paternal alleles was distorted (Fig. 1 middle column; $G_{3,\ 204\text{-}238}$ range = 138-159, P < 2.2e-16 for the two crosses). The paternal alleles S_4 and S_2 ('Vic' and 'Royalton') were the most inherited and were found in 40-50% of the offspring in the first year, and in 39-47% in the second year. On the other side, both paternal alleles of 'Cristobalina' [S_3 (5%) and S_6 (5%)] in the first year and of 'Burlat' [S_3 (9%) and S_9 (6%)] in the second year were the least inherited. Thus, following mixed-donor pollinations a particular pollen donor cultivar appears to be favoured.

To further check the influence of the genetic background, either of the female recipient or of the pollen donor, or of the environmental conditions in different years, additional crosses were carried out during a third year (lower three rows in Fig. 1). Three main observations were made. First, the replication of the cross of Year 1 ('BPv' X 'Vic':'Cristobalina') did not show the same results. All segregation analyses within each parental genotype (S_1 vs. S_5: $G_{1,\ 143} = 1.58$, $P = 0.21$, S_2 vs. S_4: $G_{1,\ 55} = 0.02$, $P = 0.89$; S_3 vs. S_6: $G_{1,\ 88} = 0$, $P = 1$) and post-pollination transmission analysis of the four paternal S-alleles were random ($G_{3,\ 143} = 7.07$, $P = 0.052$). Thus, a significant effect of the year (heterogeneity $G_{3,\ 351} = 112$, $P = 4.4e\text{-}24$) was revealed. Second, the use of different pollen donors ('Lambert' and 'Arcina') with the same four paternal *S*-haplotypes than Year 1 but in different combinations, and the same maternal genotype ('BPv'), again revealed a random segregation of both maternal and paternal alleles (S_1 vs. S_5: $G_{1,\ 112} = 1.75$, $P = 0.18$; S_3 vs. S_4: $G_{1,\ 9} = 1.02$, $P = 0.31$; S_2 vs. S_6: $G_{1,\ 103} = 1.18$, $P = 0.28$), and a skewed post-pollination transmission of the four paternal alleles in favour of S_2 and S_6 against S_3 and S_4 ($G_{3,\ 112} = 95$, $P < 2.2e\text{-}16$). Third, using a different female recipient, 'TBr' ($S_{21}S_{22}$), and the same pollen mixture 'Vic':'Cristobalina' than Year 1, again revealed a random segregation of maternal and paternal alleles (S_{21} vs. S_{22}: $G_{1,\ 93} = 0.01$, $P = 0.92$, S_2 vs. S_4: $G1,\ 80 = 0.\ 2$, $P = 0.65$; S_3 vs. S_6: $G_{1,\ 13} = 0.08$, $P = 0.78$), and a highly skewed post-pollination transmission of the four paternal alleles, again in favour of S_2 and S_4 against S_3 and S_6 ($G_{3,\ 93} = 54$, $P < 1.15e\text{-}11$). The analysis of the *S*-genotype of the offspring (Fig. 1, right column), confirmed that there were no preferential maternal-paternal *S*-allele combinations but, rather, preferential genotypic combinations (similarly significant G tests, data not shown).

Seed set and zygotic paternity ratios

Seed set ranged from 20% to 77% among the different crosses, with a variation between crosses within the same year as well as among years (Fig. S1). Compared to the first two years, the seed set registered during the third year was significantly lower (the cross as a main effect: $G_{4,\ 2144} = 762$, $P < 2.2e\text{-}16$). In the third year there was also an unexpected cold episode during the progamic phase (Table 1, minimum temperature reaching 3.2ºC, 0.3ºC at 2 and 3 days after pollination, respectively). A weekly monitoring of flower and fruit drop from pollination to fruit maturity for the first two years revealed that initial seed set was defined during the first 4 weeks following anthesis. Flower and fruit drop was indeed concentrated between the second and the fourth weeks following anthesis, and the major fruit drop occurred consistently during the third week in both years. This pattern of flower and fruit dropping, although with variable intensity, was, overall, consistent among branches within trees (data not shown), which would rule out any canopy position effect in seed set dynamics in both years.

The high seed set registered during the first two years combined with the highly skewed *S*-allele transmission raised the question of whether post-fertilisation embryo abortion alone can explain these patterns. Mathematical formulas relating these parameters for the study species (the analysis can be generalised for species having fruits with single seeds) were, thus, developed. Indeed, the analysis shows how the seed set (i.e seed survival rate $\in [0,1]$) constrains the possible values of zygotic paternity ratio for a given seed paternity ratio (Fig. 2). Three seed paternity ratios are illustrated; a hypothetical unbiased seed paternity ratio ($f = 1$), and the two seed paternity ratios obtained in the first and second years ($f = 9.4$, $f = 5.6$, respectively). On the one hand, and based on the seed paternity ratios/survival rates obtained in the crosses of the first ($f = 9.4$, $\gamma = 0.77$) and second years ($f = 5.6$, $\gamma = 0.68$), all possible z

values are illustrated with the vertical dashed lines in green and are, thus, larger than the value of the unbiased zygotic paternity ratio (z = 1, horizontal black dashed line). This finding suggests that the biased seed paternities obtained during the first two years are likely due to a biased zygotic paternity ratio ($z \neq 1$). On the other hand, under a preferential embryo abortion hypothesis (zygotic paternity ratio = z = 1), the maximum seed survival rates must not exceed $\gamma = 0.55$ in the first and $\gamma = 0.59$ in the second year (vertical red dashed line). These seed survival rate maximum values are in fact lower than the actual seed survival rates. Thus, in addition to the statistical results, the algebraic model contradicts the hypothesis of a preferential embryo abortion hypothesis, and provides support to the alternative hypothesis of a biased zygotic paternity.

Pollen performance

To evaluate whether pollen performance played a role in differential reproductive success, we characterised in detail pollen behaviour during the progamic phase for the first two years. We evaluated pollen germination at the stigma level and pollen tube growth rate in the style (Fig. 3), as well as pollen tube dynamics (pollen tube cohort size representing the inverted cone of pollen tubes) throughout the style and at the entrance of the ovary (Fig. 4).

Due to the difficulty of discriminating pollen tube identity in mixed pollinations, we compared pollen performance in single donor pollinations. At the stigma level, no significant differences were found between 'Cristobalina' and 'Vic' from pollination until 5 days later (pollen donor effect: $F_{1,\ 89} = 2.77$, $P = 0.10$, Fig. 3a). Although 'Royalton' showed significantly higher pollen germination than 'Burlat' (pollen donor effect: $F_{1,\ 86} = 5.36$, $P = 0.02$) for some days after pollination (day effect: $F_{3,\ 86} = 5.17$, $P = 8.8e\text{-}4$, Fig. 3b), pollen germination ranged between 47% and 66%, which indicated a good pollen viability for all

pollen donors tested. Similar results were obtained for all pollen donors used during the third year either in single or mixed pollinations (Fig. S2). At the style level, both 'Vic' and 'Royalton' showed, however, relatively faster pollen tube growth than 'Cristobalina' and 'Burlat', respectively, and this advantage was observed mainly during the first two days following pollination (Fig. 3c,d). Although the differences in pollen tube growth rates appear small, 'Vic' and 'Royalton' pollen tubes reached the ovary of significantly more flowers during the first day and first two days, respectively (Fig. 3e,f). These advantages could explain the relatively higher number of pollen tubes entering the ovary after 24 hours for 'Vic', and for the first three days for 'Royalton' (Fig. 3g,h). Although we did not carry out a similar pollen tube kinetic analysis for the third year, small variation was registered in the number of pollen tubes reaching the base of the style either in single or mixed pollinations (Fig. S2).

Analysing the cohort size of pollen tubes in different sections of the style for the first two crosses (Fig. 4), clearer differences could be found during the first four days following pollination. Comparing the competing pollen donors in the first cross, 'Cristobalina' and 'Vic', different behaviours were mainly found during the first two days after pollination. For the first day, although 'Cristobalina' had a larger cohort size, its pollen tube growth rate was slower, and pollen tubes were observed just at the entrance of the transmitting tissue. In contrast, 'Vic' had a smaller cohort size, but the pollen tubes already reached the first quarter of the style. A day later, a different picture emerged. Besides having a higher growth rate, the number of 'Vic' pollen tubes was higher in practically all the sections of the style. Comparing the pollen donors in the second cross, 'Royalton' had both larger cohort size in all the sections of the style (see first, third and fourth days), and faster pollen tube growth rates (see second day) than 'Burlat'. Thus, in single donor pollinations, the two genotypes that sired most of the

seeds after in mixed pollinations ('Vic' and 'Royalton'), displayed higher pollen tube growth rates, higher cohort sizes, and reached each level of the style and the ovary entrance earlier.

DISCUSSION

Paternal-specific distorted transmission of the *S*-alleles after mixed donor pollinations

In the different compatible mixed-donor pollinations, our results revealed that, while gamete or embryo sorting within any given pollen donor or female recipient was random, a paternal-specific non-random sorting among gametes or embryos occurred in most of the crosses.

Previous reports using 'Cristobalina' pollen in single donor fully compatible pollinations with other maternal cultivars also revealed random segregation of all genotypes (Wünsch & Hormaza, 2004a; Cachi *et al.*, 2014). Thus, the distortion obtained in this work after mixed-donor pollinations would not be related to an inherent lower transmission ratio of the individual haplotypes. However, in some specific single donor pollination experiments in sweet cherry (Ikeda *et al*., 2004; Wünsch & Hormaza 2004b), and apricot (Vilanova *et al*., 2006), where pollen-self-compatible pollen donors share alleles with the maternal genotype, a non-random segregation has been observed; Cachi et al. (2014) have proposed S-haplotype associated differential pollen tube competitivity as a possible explanation. Linkage or linkage disequilibrium with pollen competitive ability genes, or mutational load hitchhiked within the non-recombining region around the *S*-locus (Charlesworth, 2006), or mutations in SI modifier genes (Cachi *et al.*, 2014) are other potential mechanisms. The results obtained herein after mixed-donor pollinations suggest further possibilities. When combined together, in three out of five crosses, S_2 and S_4 *S*-alleles were the most competitive whereas S_3, S_6 and S_9 were the least. Although the use of a different female recipient appears to have no effect on these

transmission distortions, replicating one of the crosses during an unexpected cold episode or using other pollen donor genotypes, with the same paternal *S*-haplotypes but in a different genotype combination, appears to affect the consistency of the *S*-haplotype competitive behaviour. Our results point to the identity of the pollen producing plant and to environmental conditions as potential buffering mechanisms.

The potential for pre-zygotic sexual selection

Paternal-specific *S*-allele transmission suggests that sexual selection, mediated by prezygotic preferential fertilization or postzygotic selective embryo abortion, might be occurring in this species. Working with wild radish, and after avoiding the possible interference of the self-incompatibility reaction by performing fully compatible mixed-pollination regimes, Marshall (1998) found a consistent non-random mating (non-modulated by the female genotype), which is a clear indicator for the occurrence of sexual selection. Although our results also support this hypothesis, a female choice component cannot be ruled out without characterising additional S-allele specificities.

The proportion of flowers that became fruits with seeds, and, hence, definitive paternity establishment, was essentially defined during the first three to four weeks after pollination. In sweet cherry, the progamic phase is temperature dependent and usually lasts between 4 and 8 days, and fruits mature between 8 and 9 weeks after pollination (Hedhly *et al.*, 2007). Thus, these results point to an early event encompassing the progamic phase or early embryo development that establishes paternities. On the other hand, the mathematical relationship between zygotic paternity ratios, seed paternity ratios and seed survival rates reveals that the observed, highly skewed transmission patterns for the levels of seed survival rates are incompatible with embryo abortion as the main underlying mechanism. However, embryo

abortion cannot be excluded as an explanation during the third year when a lower seed set was registered; the cold episode that occurred during the pollination-fertilisation phase (when minimum temperatures reached 3.2ºC and 0.3ºC at 2 and 3 days after pollination, respectively) has probably affected the fertilisation level. Overall, our results further narrow down the developmental window most likely responsible for the distorted transmission patterns to the progamic phase before fertilization.

In wild sweet cherry, *S*-allele frequencies were found to vary within and between populations, and to depart from the expected isoplethic distribution likely due to population subdivision or genetic drift (De Cuyper *et al.*, 2005; Stoeckel *et al.*, 2008). Based on our results, we propose that post-pollination pre-fertilization sexual selection is potentially another selective pressure. Mixed donor loads on wild sweet cherry stigmas would be a prerequisite for such a selective force to have an effect, and this is likely to occur since high pollen flow distances and high polyandry have been reported under natural conditions (Jolivet *et al.*, 2011). Interestingly, unequal paternal contribution has also been reported in this species (Jolivet *et al.*, 2011).

Sexual selection mediated by gametophytic behaviour

Post-pollination pre-zygotic sexual selection requires male-male competition or male-female differential interaction (i.e. female choice) (Willson & Burley, 1983). In our detailed characterisation of pollen performance for the first two years, both pollen donors siring most of the seeds showed consistently higher pollen tube growth rates in the pistil and larger pollen tube cohorts at different sections of the style. Although this behaviour is in fact registered in single donor pollinations, it is probably decisive under mixed pollination, where pistil

reserves are limited for a number of pollen tubes (Herrero and Hormaza, 1996), and where only one ovule is to be fertilized by a single male gametophyte.

Taking into account that the use of a different female recipient during the third year produced the same type of transmission distortions, and one paternal parent was preferentially fertilizing, these findings point to pollen-pollen competition as a mechanism underlying differential siring success. Similar results pointing to pollen competition as an explanatory mechanism of skewed paternity derived from intraspecific crosses using mixed-donor pollinations have been obtained using tetraploid and diploid *Chamerion angustifolium* individuals (Husband *et al.*, 2002). The tetraploid donor had more pollen tubes at different sections of the style in single donor pollinations and sired most of the seeds after mixed pollination both in its own pistils and in those of the diploid recipient, 70% and 83% respectively. However, in an interspecific mixed pollination experiment implicating diploid *Betula occidentalis* and hexaploid *B. papyrifera,* where conspecific pollen sired more than 98% of the offspring, this skewed paternity seemed to be mediated rather by favourable male-female interactions than by male-male competition (Williams *et al.*, 1999). But, in contrast to our results, both studies might reflect specific cases of non-random mating as a consequence of different mate sorting mechanisms (see Marshall, 1998; and Marshall & Diggle, 2001 for a discussion on the subject). Additional crosses with other *S*-allele specificities would definitely clarify whether *S*-allele identity itself is related to pollen tube growth or to male female-interaction in a compatible pistil.

Our results provide evidence for the occurrence of paternal-specific *S*-haplotype transmission distortion. Based on the comparative analysis between *S*-allele segregations and their post-pollination transmissions after mixed pollinations, on seed set dynamics and on pollen performance, we could narrow down the developmental window most likely responsible for these distorted transmission patterns to male-male pollen donor competition -

i.e. post-pollination pre-fertilization sexual selection. In summary, these results support the hypothesis of post-pollination sexual selection as a selective force tempering frequency dependent selection; and they suggest novel mechanisms underlying unequal *S*-allele frequencies in natural populations.

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Table and Figure captions

Table 1. Mixed donor pollination experiments, their *S*-genotypes, and environmental conditions (Maximum, Average, and Minimum temperature and relative humidity) registered during the first 7 days after pollination.

Fig. 1 *S*-allele segregations and transmission. Maternal (left column) and paternal (middle column) *S*-allele segregations/transmission, and offspring *S*-allele genotypes (right column) in the different crosses performed the first (upper row), second (second row) and third year (bottom three rows). Seed paternities are highlighted in grey within the third column. Expected percentages for random segregation of maternal *S*-alleles (50% for each maternal alleles, black line), and approximate transmission percentage of each of the four paternal *S*-alleles (25% solid grey), of paternity (50%, dashed black), and of S-genotypes (12.5%, dashed grey) are highlighted; actual expected transmission values are corrected for observed segregation values and vary little above and below these values.

Fig. 2 How seed set and seed paternity levels constrain zygotic paternity. Maximum and minimum zygotic paternity ratios as functions of seed set (survival rate, γ), for three given values of seed paternity ratios (f = 1: unbiased seed paternity; f = 9.4 and f = 5.6: the obtained paternity ratios during the first and the second year respectively). z_{max} is the upper border in each filled area; z_{min} is the bottom border in each filled area. The unbiased zygotic segregation ratio (z = 1) is indicated with the horizontal black dashed line. For a given value of f, any combination (γ, z) that lies outside the filled area is not possible. For f = 1, note how

an unbiased fertilization (z = 1) for any seed survival rate level cannot be excluded. Under an unbiased zygotic segregation ratio (z = 1), the theoretical maximum seed survival rates for both biased seed paternity ratios are indicated with the vertical dashed lines in red. For the obtained survival rate (77.1% and 67.7%), the range in which the zygotic segregation ratio can lie is indicated with the vertical dashed lines in green; note that in both cases the unbiased zygotic segregation ratio lies outside the possible range.

Fig. 3 Pollen germination and pollen tube kinetics after single donor pollinations. Percentage of pollen germination at the stigma (a, b), length of the longest pollen tube as percentage of the length of the style (c, d), percentage of flowers with pollen tubes at the base of the style (e, f), and number of pollen tubes reaching the ovary (g, h) throughout the first 5 days following pollination for the crosses performed the first two years. Error bars represent standard errors (se).

Fig. 4 Pollen tube dynamics after single donor pollinations. Pollen tube cohort sizes throughout the stigma-style-ovary (tt: transmitting tissue of the style) during the first four days following pollination for the crosses performed the first ('BPv' x 'Vic':'Cristobalina') and second ('BPv' x 'Royalton': 'Burlat') years. For each day, half the number of pollen tubes (and respective se bars) are displayed at each side of the central vertical line in such a way (x-axis origin at the center and scale increasing outward at both sides) that a schematic representation of the typical inverted cone of pollen tubes growing down the style can be appreciated.

Table 1 Mixed donor pollination experiments, their *S*-genotypes, and environmental conditions (Maximum, Medium and Minimum temperature and Relative Humidity) registered during the first 7 days after pollination.

			Temperature ºC (RH%)		
Year	Cross	S-genotypes	Max.	Med.	Min.
1	'BPv' X ('Vic':'Cristobalina')	S_1S_5 x S_2S_4: S_3S_6	22.7 (94)	14.4 (69)	6.5 (35)
2	'BPv' X ('Royalton':'Burlat')	S_1S_5 x S_2S_4: S_3S_9	19.8 (94)	13.6 (77)	8.4 (50)
3	'BPv' X ('Vic':'Cristobalina') 'BPv' X ('Lambert':'Arcina') 'TBr' X ('Vic':'Cristobalina')	S_1S_5 x S_2S_4: S_3S_6 S_1S_5 x S_3S_4: S_2S_6 $S_{21}S_{22}$ x S_2S_4: S_3S_6	17.7 (95)	11.5 (74)	5.6*(42**)

* Minimum temperature of this year reached 3.2ºC, 0.3ºC and 2.5ºC at 2, 3, and 7 days after pollination, respectively.

** The Relative Humidity (mainly the Max) also showed a sharp reduction 2 and 3 days after pollination.

Supporting information

Materials and Methods

Fig. S1

Fig. S2